Exosome-mediated miR-21 promotes angiogenesis within esophageal tumor microenvironment by activating PTEN/Akt signaling pathway in Vascular Endothelial Cells

Tian Wang

Southeast University

Juan Liao

Hangzhou First People's Hospital

Zijie Yang

Southeast University

Weitao Shen

Southeast University

Zhi kui Gao

Southeast University

Jie chen Yin

Southeast University

Li hong Yin

Southeast University

Ran Liu (✉ ranliu@seu.edu.cn)

Southeast University

Research

Keywords: Exosome, miR-21, Angiogenesis, PTEN/Akt, Esophageal tumor microenvironment

Posted Date: May 29th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-31065/v1>

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Abstract

Background

Angiogenesis, a pivotal component in the tumor microenvironment (TME), boosts tumor growth and metastasis. Cancer-derived exosomes, which have been widely reported to play a crucial role in the establishment of TME can be effective angiogenic modulators. We aim to investigate the contribution of microRNA-21 (miR-21) for angiogenesis which was packaged in cancer-derived exosomes in esophageal squamous cell carcinoma (ESCC).

Methods

The co-cultivation model was constructed to mimic the tumor microenvironment based at a physical level to explore the effects of cancer-derived exosomes on angiogenesis of human umbilical vein endothelial cells (HUVECs). EdU assay, transwell assay and tube formation assay formation experiments were conducted for the evaluation of HUVECs proliferation, migration, and angiogenesis, respectively. In addition, Dual-luciferase reporter (DLR) assay was performed to validate the relationship between miR-21 and its target gene PTEN. Similarly, miR-21 inhibitors and LY294002 was applied to evaluate the regulation of miR-21 via pro-angiogenesis in recipient HUVECs by PTEN/Akt signaling pathway.

Results

After 24 h co-cultivation with EC9706 cells, miR-21 levels in recipient HUVECs was raised. The results from EdU assay, transwell assay and blood vessel formation experiment showed that exosomes which were secreted from EC9706 cells (EC9706-Exo) delivered miR-21 stimulated proliferation, migration and tube formation of HUVECs. DLR assay indicated that miR-21 could directly bind to the 3'-untranslated region (UTR) of PTEN genes, real-time PCR and western blot analysis for PTEN showed it was inhibited by EC9706-Exo shuttled miR-21. Meanwhile, phospho-Akt (p-Akt) (Ser473), one of the downstream genes of PTEN, was significantly increased in recipient HUVECs compared to the control group, while inhibiting miR-21 and PI3K/Akt pathway respectively both led to a sharp decrease in p-Akt levels, suggesting that exosomal miR-21 promote angiogenesis via activating PTEN/Akt signaling pathway.

Conclusion

Exosomal miR-21 acts as a driver of pro-angiogenesis by activating PTEN/Akt signaling pathway, it might serve as a blood-based biomarker for ESCC metastasis. Suppressing the expression or blocking the transmission of these exosome-derived miR-21 might be a novel antiangiogenic therapeutic strategy for ESCC.

Background

Esophageal carcinoma (EC) is the seventh most common cancer (572,000 new cases) and the sixth in mortality worldwide (509,000 deaths) in 2018(1). China comprised nearly half of new cases, deaths, and disability-adjusted life years (DALYs) globally in 2017(2). Moreover, one of the main subtypes of EC—esophageal squamous cell carcinoma (ESCC), comprises over 90% of EC in China (3, 4). EC is one of the most difficult malignancies to cure(5). Although the combination of surgery, radiotherapy and chemotherapy has improved, the results of EC have been disappointing, especially the metastatic ESCC(6). Controlling EC metastasis effectively may prolong the survival time of EC patients. Tumor metastasis is affected by a variety of factors, yet emerging evidence showed angiogenesis contributes greatly to it(7–9). However, cancer progression is more than just changes in the cancer cells themselves as changes in the tumor microenvironment (TME) have also been shown to play a critical role in all stages of tumorigenesis (9). The poor functionality of tumor blood vessels has profound consequences for the TME and can lead to hypoxia, decreased immune cell infiltration and activity, and increased risks of metastatic dissemination(10). Further exploration of the molecular mechanism of tumor angiogenesis in TME may open up new possibilities for therapeutic strategy. One of the relative mechanisms is based on exosomes. As an important component of the TME, exosomes have an critical impact on angiogenic programs which is launched by hypoxia-induced cell signaling in cancer cells.

Exosomes are small extracellular vesicle in a size range of 40–160 nm in diameter with an endosomal origin, playing a part in the development, invasion, migration, metastasis and drug resistance of cancer(11–13). They are produced by various cells, but tumor cells are especially active exosome producers, potentially because the presence of stress or hypoxia stimulate tumor cells to increase exosome production(14). Tumor cells secrete millions of exosomes and distribute them throughout the TME. Patients with cancer, especially those with advanced or metastatic disease, have greatly increased numbers of exosomes in the plasma compared to healthy blood donors(15). Exosomes contain a variety of biologically active molecules, such as microRNAs (miRNAs), messenger RNAs (mRNA), DNA fragments and proteins (16, 17), which can be transferred to remote sites to regulate the function of distant cells and may affect the processes of receptor cells, especially by promoting interaction between various cells in the TME(18–20). Exosomal miRNAs have been emphasized a lot, which can make the TME more suitable for tumor development by promoting endothelial cells to form tubes, which is conducive to the metabolism and survival of tumor cells. During the progression of the tumor, primary tumor-derived exosomal miRNAs can be transferred to non-malignant cells in the TME to induce heterogeneity(21–23). At the same time, with the changes in biological activity of non-malignant cells in the TME, non-malignant cells can also secrete exosomal miRNAs to further regulate tumor cells or other microenvironmental components(24, 25). They also mediate inflammatory cell infiltration and immune escape, which is conducive to colonization and proliferation of tumor cells. Thus, tumor cells can reshape their own microenvironment through paracrine, promoting tumor growth and metastasis.

Our previous studies showed that microRNA-21 (miR-21) was abundant in both EC cells and their corresponding exosomes(26). MiR-21 is reported to be over-expressed in various tumors, including

esophageal cancer, breast cancer, gastric cancer, colorectal cancer, pancreatic cancer lung cancer and so on(27–31). These studies also proved that miR-21 has multiple functions in human cancers, including promoting cell proliferation, migration, invasion, metastasis, suggesting that miR-21 functions as an oncogene and modulates tumor growth and development. Thus, it may serve as a novel therapeutic target.

In this current research, we found the biological functions of human vascular endothelial cells (HUVECs) were significantly enhanced by coculture with EC cells, while the expression of PTEN protein in HUVECs was obviously decreased. Bioinformatics analysis combined with luciferase assays indicated that miR-21 directly targets the 3'-untranslated region (UTR) of PTEN mRNA. Then, we verified the phenomenon above by the upregulation of miR-21 directly in HUVECs, which demonstrated that overexpression of miR-21 can promoted the growth and angiogenesis of HUVECs. We also explored the regulatory mechanism behind PTEN by upregulation or downregulation of miR-21, including the angiogenesis-related genes and downstream molecules. Therefore, our study reveals a novel mechanism of angiogenesis in ESCC, and it supports the hypothesis that the level of miR-21 in exosomes can be a potential biomarker for evaluating invasion or metastasis in ESCC.

Materials And Methods

Cell culture

The human esophageal cancer cell line EC9706 (National Laboratory of Molecular Oncology, Cancer Institute, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China) was cultured in RPMI-1640 (Gibco, USA) medium. HUVECs (Shanghai Institute of Biochemistry and Cell Biology, CAS) were cultured in F12K medium (Gibco, USA). Both of the basal culture media above were supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin and streptomycin. FBS was centrifuged 10,000× g for 30 min, followed by ultracentrifugation at 200,000×g for 6 h to eliminate bovine-derived exosomes using a Type 70 Ti rotor in L-80XP ultracentrifuge (Beckman Coulter, Brea, Ca, USA). Cells were incubated in a humidified incubator at 37°C with 5% CO₂.

Isolation of exosomes from medium

Exosomes were isolated from cell culture medium after a 48-h of culture by differential centrifugation according to previous studies(32). Briefly, the medium was first centrifuged at 300 × g for 10 min, 800 × g for 10 min, 1,200 × g for 20 min, and 10,000 × g for 30 min to remove any live or dead cells or cellular debris. Afterwards, the supernatant was ultracentrifuged at 100,000 × g for 3 h to pellet exosomes. Finally, the supernatant was disposed, and the exosome pellet was washed with phosphate-buffered saline (PBS) at 100,000 × g for 2 h. All steps were performed at 4°C. Exosomes were harvested from the pellet and resuspended in PBS. The exosome levels were determined by measuring the total protein content, which was presented as micrograms of total protein in the exosomes. The exosome fraction was

measured for its protein content using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Wilmington, DE, USA).

Exosome labeling and transmission electron microscopy assay

The assay of exosome labeling and live-cell fluorescence microscopy as previously reported, firstly, the exosome pellet suspension was diluted with RPMI-1640 medium by adding Dil. Then, 2×10^5 EC9706 and 1.5 ml complete medium were placed in a 35-mm glass-bottom culture dish for Live Cell Imaging System analysis. After a 24 h culture, the Dil-labeled exosomes were incubated with cells for 2.5h and washed once to eliminate the free exosomes. Cells were then transferred to the cell culture chamber of the microscope.

Cell transfection

HUVEC cells were seeded into 6-well plates and transfected using Lipofectamine RNAiMAX (Invitrogen) and Opti-MEM (Gibco, USA), followed by the manufacturers' instructions. For miRNA upregulation and downregulation, a 50-nmol dose of miR-21 mimics, inhibitors, and NC was used. Cells were harvested 36h after transfection to isolate total RNA or total cell lysate. Transfer efficiency was determined as fluorescent cell percentage by flow cytometry. The miR-21 expression level was detected by real-time quantitative polymerase chain reaction(RT-qPCR). EC9706 cells were cultured in 100-mm dishes and transfected with miR-21 mimics for the ready of co-cultivation model.

Construction for co-cultivation model

Co-cultivation of donor EC9706 cells and recipient HUVECs were performed in 12-well transwell inserts (cat. no. 3401; Corning Inc., Corning, NY, USA). HUVECs were pre-seeded in the lower chambers at a 1×10^5 cells/well density. Then, the EC9706 cells, transfected with miR-21 mimics or the negative controls, were scraped off and seeded onto 0.4 μ M transwell inserts in the following day.

RNA extraction (isolation) and RT-qPCR

Total RNA was extracted from the cultured cells and exosomes using Trizol reagent (Invitrogen) and mirVana miRNA isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's protocols, respectively. RNA concentration was analyzed using NanoDrop spectrophotometer (NanoDrop ND-1000; NanoDrop Technologies, Inc., Wilmington, DE, USA). After the reactions were complete, the cycle threshold (CT) data were determined using fixed threshold settings, and the mean CT values were determined from triplicate PCRs. We used the formula to calculate the relative quantities of target genes. RNU6 was used as the internal control for cells and supernatant, respectively, in miR-21 expression level analysis. β -actin was used as the invariant control for mRNA analysis. The sequences of PTEN primers were as follows: forward, 5'-AATGGCTAAGTGAAGATGACAAT-3' and reverse, 5'-TGCACATATCATTACACCAGTTCGT-3'. The sequences of matrix metalloproteinases (MMP)-2 primers were as follows: forward, 5'-CTGATGGCACCCATTTACA CCT-3' and reverse, 5'-GATCTGAGCGATGCCATCAAA-3'. The sequences of

MMP-9 primers were as follows: forward, 5'-TGGGCTACGTGACCTATGACAT-3' and reverse, 5'-GCC CAGCCCACCTCCACTCCTC-3'. The sequences of β -actin primers were as follows: forward, 5'-ATCCGCAAAGACCTGT-3' and reverse, 5'-GGGTGTAACGCAACTAAG-3'. The primers used for the amplification of miR-21, RNU6 and cel-miR-39 were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The PCR reaction was performed at 95°C for 5 min, which was followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec. Dissociation curve was analyzed from 60 to 99°C. Relative transcript quantities for each miRNA were calculated using the $\Delta\Delta C_t$ method.

The miRNA target prediction and luciferase reporter assay

The miRNA target prediction and analysis were performed with the algorithms from DIANA (<http://diana.imis.athena-innovation.gr/DianaTools/index.php>), TargetMiner (https://www.isical.ac.in/~bioinfo_miu/targetminer20.htm), and miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/>), RNA22 (<https://cm.jefferson.edu/rna22/Interactive/>). The reporter plasmid pmiR-PTEN was designed by RiboBio (Guangzhou, China). EC9706 cells in the miR-21 mimics or negative control group were transfected in 96-well plates (1×10^4 cells/well) with pmiR-Report-WT-PTEN by Lipofectamine RNAiMAX. The renilla luciferase was used as an internal control. At 48 h after transfection, cells were analyzed using a Dual Luciferase Assay Kit (Promega).

Vascular ring formation by HUVECs in vitro angiogenesis assay

An in vitro angiogenesis assay was performed as previously described(33, 34). Briefly, 30 μ l Matrigel (BD Biosciences) was added to each well of a 96-well plate and allowed to polymerize at 37°C for 30 min. Next, the pretreated HUVECs were resuspended in FBS-free RPMI-1640 medium and transferred to each well at a concentration of 4×10^4 cells per well. After 12 h of culture in a humidified incubator at 37°C with 5% CO₂, the cells were examined under an Olympus FSX100 light microscope (Tokyo, Japan) to assess the formation of capillary-like structures. The total tubule length, number of closed meshes and branch points of the formed tubes, which represent the degree of angiogenesis in vitro, were scanned and quantified in at least ten low-power fields (40 $\times$ magnification).

Cell proliferation assay

The proliferative ability of HUVECs after different transfections or different exosome cocultures was determined by Cell-Light EdU Apollo in vitro image kit (RiboBio, Guangzhou, China). After pretreatment as described above, HUVECs were incubated in 50 μ M EdU for 2 h, and they were then fixed, permeabilized, and stained following the appropriate instructions.

Cell migration assay

The migratory capacity of HUVECs was performed using a transwell insert that contains a polycarbonate filter with 8 μ m pore size (cat. no. 3422; Corning). The pretreated HUVECs cells (5×10^3 /well) suspended in 150 μ l serum-free RPMI-1640 were added to the 24-well upper chamber, and 600 μ l RPMI-1640 that

contains 10% FBS was added to the bottom wells of the multiwell insert assembly. Cells were incubated at 37°C for 24 h to allow cell migration through the membrane. Migrated cells were fixed in 95% ethanol and stained with crystal violet. The number of invaded cells was counted under Olympus FSX100 light microscope (Tokyo, Japan). To minimize the bias, cells in ten randomly selected fields at a 200×magnification were counted to calculate the average cell number.

Western blot analysis

The expression of PTEN was assessed by western blot analysis, and its expression in the samples was normalized to β -actin expression. Cells were lysed in RIPA buffer with freshly added protease inhibitor. Total lysates were separated on SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). The immunoblots were blocked with 5% fat-free milk and they were incubated at 4°C overnight with primary antibodies anti-PTEN (1:1000, Cell Signaling Technology), anti- β -actin (1:1000, BM0627; Wuhan Boster Biological Technology, Ltd., Wuhan, China). Akt (Ser473) (1:1000, Cell Signaling Technology) phosphorylation were examined by using phospho-specific antibodies. Total protein was determined using anti-Akt antibodies. After incubation with the secondary antibody, the membranes were visualized with an enhanced chemiluminescence system kit (Millipore, USA), according to the manufacturer's protocol. To explore whether miR-21 promote angiogenesis in tumor microenvironment by PTEN/Akt signaling pathway, the recipient cells were treated PI3K/Akt inhibitor LY294002 which was obtained from Beyotime Biotechnology (Nantong, China) with a final concentration of 60 μ M.

Assessment of nitric oxide (NO)

Total nitric oxide concentration in the supernatant of HUVECs medium was detected by measuring the concentration of nitrate and nitrite according to Nitric Oxide Assay Kit by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The optical densities at 550 nm wavelength were recorded using an ELISA reader (BioTek Epoch, USA) and the concentrations of NO were calculated according to the standard curve.

Statistics

All values are expressed as mean + SEM unless otherwise noted. The results are presented as the average of at least three experiments, each performed in triplicate, with standard errors. Data were described with median values $\pm$ SEM and analyzed by using Student's t test for 2-group comparisons. Differences were considered statistically significant at $P < 0.05$. In this study, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Results

Exosomes transport Cy3-miR-21 from EC9706 into HUVECs

To determine whether exosome-delivered miR-21 can be shuttled into HUVECs and the effects of it on angiogenesis of HUVECs, Cell co-cultivation was performed using a 12-well transwell as described in Materials and methods. After co-cultivation with donor EC9706 cells which was successfully transfected

with Cy3-labeled miR-21 for 24 h, recipient HUVECs were in red fluorescence with a large proportion under fluorescence microscope (Fig. 1a). RT-qPCR results showed that the expression level of miR-21 in miR-21 mimics-transfected group was higher than the negative control group, with an average fold change of 1.23 in recipient cells (Fig. 1b) after 24 h of culture. The results showed that miR-21 was suggested to be secreted from donor EC9706 cells and was delivered into recipient HUVECs cells via exosomes.

Exosome-shuttling miR-21 promotes HUVECs angiogenesis activities

We further evaluated the effects of miR-21 from EC9706-Exo on angiogenesis in vitro by performing a tube formation assay. To examine the effect of miR-21 on angiogenesis, primary HUVECs were treated with miR-21 mimics and NC mimics for 48 h. After then HUVECs were seeded on the surface of Matrigel and incubated at 37 °C for 12 h, tube formation of HUVECs (Fig. 2a) was significantly enhanced in the miR-21 mimics treated group as determined by the increase of the tube length (Fig. 2b), the number of closed meshes (Fig. 2c) and the number of junctions (Fig. 2d), with an average fold change of $2.11 \pm 4.83 \pm 2.13$ (all P -value < 0.05) in comparison to control group. After coculture with EC9706 exosomes and NC exosomes for 24h, the tube length (Fig. 2f), the number of closed meshes (Fig. 2g) and the number of junctions (Fig. 2h) in HUVECs (Fig. 2e) were increased than that in control group (all P -value < 0.05). We demonstrated that, in contrast to NC exosomes, EC9706 exosomes could promote HUVECs angiogenesis in vitro.

Exosome-shuttling miR-21 promotes the proliferation and migration of HUVECs

To further confirm the impact of miR-21 on the biological behavior of HUVECs, miR-21 mimics, NC mimics transfected directly into HUVECs. Cell proliferation (Figures. 3a and 3b) and cell migration (Figures. 3c and 3d) were increased by 27.65% and 44.41% respectively in primary HUVECs treated with miR-21 mimics than that in miR-NC group. While HUVECs cocultured with EC9706-Exo exhibited increased cell proliferation by 18.24% (tested by an EdU proliferation assay) (Figures. 3e and 3f) and cell migration by 53.20% (assessed by a transwell chamber migration assay) (Figures. 3g and 3h). These results remind us that EC9709-exo delivered miR-21 had been shown to contribute to promoting tumor growth and metastasis in the simulated TEM based on the coculture model at a physiological level.

Exosome-delivered miR-21 suppresses PTEN by directly binding to its 3'-UTR in HUVECs

Based on the predictive results of our bioinformatics analysis, we preliminarily confirmed that miR-21 can directly target the 3'-UTR of PTEN mRNA in a highly conserved manner among species (Figure. 4a). In addition, a luciferase assay showed that the relative luciferase activity was clearly inhibited when miR-21 mimics were co-transfected with the luciferase reporters (Fig. 4b). However, the interaction was lost in NC group. These results indicated that miR-21 can directly bind the 3'-UTR of PTEN genes.

We performed qRT-PCR and western blot analysis to detect the PTEN mRNA and protein level in recipient HUVECs after a 24h coculture. PTEN mRNA levels in exosome-shuttling miR-21 group was significantly lower (0.54 fold) than that in the NC group (Fig. 4c), while the miR-21 inhibitor transfection increased

PTEN mRNA level to 1.6 fold than the miR-21 group. Moreover, WB analysis revealed that exosomes from miR-21-transfected EC9706 cells led to a sharp reduction (43.71%) in PTEN expression under the co-cultivation model (Fig. 4d and 4e). These results confirmed EC9706-Exo mediated miR-21 targeted PTEN directly.

Exosome-shuttling miR-21 effect on p-Akt (Ser473) expression in recipient HUVECs

Compared with miR-NC groups, phospho-Akt (p-Akt) (Ser473), one of the downstream genes of PTEN, was significantly increased by 40.00% (Fig. 5) in HUVECs after coculture with EC9706 cell. While inhibiting PI3K/Akt pathway and miR-21 led to a sharp decrease by 51.68% and 49.93% respectively in p-Akt levels. These results indicated that EC9706-Exo delivered miR-21 could target PTEN/Akt signaling pathway directly in HUVECs.

Exosome-shuttling miR-21 effect on the level of NO of recipient HUVECs

We evaluated NO production of supernatant in recipient HUVECs which were cocultured with donor EC9706 cells for 24hr. NO increased by more than 1.8 fold in miR-21 group than that in miR-NC group ($P < 0.05$; Fig 6). But we didn't observe a decline of NO levels when incubated with LY294002. That may be due to the NO content in the culture supernatant of HUVECs is too low to be distinguished by the current method in our study.

Discussion

Emerging evidences showed tumor growth is angiogenesis dependent. Angiogenesis, the recruitment of new blood vessels, is also an essential component of the metastatic pathway(7). Both expansion of the primary tumor and metastasis to distant organs depend critically on the formation of new blood vessels that provide increased availability of oxygen and nutrients to the tumor (7). Thus it has been well recognized that most solid tumors contain large numbers of highly permeable blood vessels (1), allowing them to develop their own supplies of nutrients and oxygen and enabling their growth and metastasis. Hypoxia is one of the main driving forces of tumor angiogenesis. It triggers vessel growth by signaling through hypoxia-inducible transcription factors in tumor tissue. When cancer cells are exposed to hypoxia, the exosome production increases and leads to increased levels of signaling in the TME. Recently, the ability of tumor-derived exosomes (TEX) to induce angiogenesis via modulating mechanisms responsible for the blood vessel development in various cancers and TME has been well documented. For instance, Glioblastomas are highly vascularized in comparison to other solid tumors(35). They produce TEX, which influence the proliferation of endothelial cells. Exosomes secreted by glioblastomas contain angiogenic proteins and have pro-angiogenic properties both in vitro and in vivo (36, 37). Other solid tumors, such as colorectal cancer, breast cancer or pancreatic carcinoma, also produce exosomes that induce angiogenesis (18–20). A related mechanism implicated in angiogenesis by exosomes involves transfer of miRNAs which can mediate angiogenic effects in various types of tumors. Zeng et al. reminded that cancer-derived exosomal miR-25-3p might be an effective biomarker for colorectal cancer metastasis by angiogenesis(38). Yang et al. demonstrated that exosomes delivered

miR-130a from gastric cancer cells into vascular cells to promote angiogenesis and tumor growth by targeting c-MYB both in vivo and in vitro(39). The critical role of angiogenesis via TEX in promoting tumor growth and metastasis is strongly established(40, 41).

Previous studies from our laboratory revealed that miR-21 was highly expressed in the plasma from human ESCC patients(42). That research found tumor-derived miR-21 promotes cell migration and invasion in EC. In the present study, we visualized the transfer of EC9706-Exo derived miR-21 to HUVECs by constructing coculture model at a physical level, and we found that exogenous miR-21 via exosomal transport could function as an endogenous miRNA in endothelial cells, we proved that cell-to-cell communication via exosomal miR-21 affected endothelial migration and tube formation. miR-21 has been widely studied and recognized as an oncogene in promoting the progress of various cancers, such as gastric cancer(43), breast cancer(44), lung cancer(45), EC(46). Concerning the role of miR-21 in angiogenesis, several reports find that miR-21 functions as a regulator of angiogenesis. Zhou et al. demonstrated that overexpressing miR-21 could improve neovascularization in critical limb ischemia by targeting CHIP to enhance HIF-1 α activity(47). Du et al. indicated that increased miR-21 expression promoted proliferation and angiogenesis of endothelial progenitor cells via targeting FASLG(48). Another research found that andrographolide inhibits angiogenesis by inhibiting the miR-21/TIMP3 signaling pathway(49). Furthermore, Liu et al. found exosomal miR-21 from transformed human bronchial epithelial cells promotes angiogenesis regulated by STAT3, providing a new perspective for intervention strategies to prevent carcinogenesis of lung cancer(50).

Although regulation of proliferation, epithelial-mesenchymal transition, invasion, and tumor angiogenesis attributed to miR-21 is studied in a variety of cancer types(51, 52), the specific function of exosomal miR-21 via pro-angiogenic activity in ESCC have never been elucidated. Our data showed that EC9706-Exo delivered miR-21 enhanced recipient HUVEC cell migration and tube formation in a simulated TME. Additionally, we identified PTEN as a target of miR-21 in mediating angiogenesis. As we all known, PTEN is the inhibitor of PI3K/AKT signaling pathway(53, 54). It has been widely reported to inhibit angiogenesis via negative regulation of PI3K/Akt signaling pathway. Cheng et al. indicated that astragaloside IV could promote angiogenesis by activation of PTEN/PI3K/Akt signaling pathway(55). Ma et al found that siRNA-mediated inhibition of PTEN gene expression in pancreatic cancer cells increased p-Akt activation, up-modulated the proliferation, and migration of cocultured vascular endothelial cell and enhanced tubule formation by HUVEC, but this function was clearly decreased by LY294002 and Akt inhibitor in PTEN siRNA transfected cells(56). Another study also showed metformin-induced anti-angiogenic effects through targeting PTEN and SMAD7 expression and PI3K/Akt pathway(57). In our study, we proved that p-Akt, one of the downstream genes of PTEN, was up-regulated in recipient HUVECs after coculture with EC9706 cells, while inhibiting PI3K/Akt pathway and miR-21 respectively both led to a sharp decrease in p-Akt levels, suggesting that EC9706-Exo delivered miR-21 increased the activity of Akt signaling via down-regulating PTEN.

Besides, NO, one of the downstream molecular of Akt-endothelial nitric oxide synthase (eNOS) has been testified to promote angiogenesis in endothelial cells or others (58, 59). It has been recognized that NO

enhances endothelial cell migration, tube formation, and proliferation, and protects endothelial cells from apoptosis [23, 24]. Our data found that EC9706 delivered miR-21 led to the increased levels of NO production in recipient HUVEC cells, but we didn't observe a decline of NO levels when incubated with LY294002. That may be due to the NO content in the culture supernatant of HUVECs is too low to be distinguished by the current method in our study.

There are several limitations in this study. Firstly, this research was only performed in vitro, however, it is unclear whether the same effects can be achieved in vivo, so the in vivo role of exosome-derived miR-21 in angiogenesis and tumor growth needs to be confirmed. Secondly, the research mechanism of angiogenesis in ESCC is not deep enough. For instance, eNOS, one of the downstream molecules of Akt, produces NO, and enables a long-term proliferative response, contributing a lot to angiogenesis. The eNOS pathway is also mediated by vascular endothelial growth factor (VEGF), a pro-angiogenic species that is often targeted to inhibit tumor angiogenesis. Further study needs to be explored in the future. Moreover, the clinical relevance of exosomal miR-21 and PTEN expression in the plasma of ESCC patients awaited further validation. In summary, our findings provide novel insights into the intercellular communications between ESCC cells and HUVECs in TME. In view of the crucial status of angiogenesis in tumor formation and progression and the single therapeutic strategy of existing antiangiogenic drugs, it is important to find new anti-vascular targets. MiR-21 might be a novel strategy for anti-angiogenic therapy in ESCC.

Conclusions

Our data demonstrate that miR-21 enclosed in exosomes secreted from cancer cells acts as a driver of angiogenesis by regulatory of PTEN/Akt pathway, suppressing the expression or blocking the transmission of these exosome-miR21 might be a novel antiangiogenic therapeutic strategy for ESCC.

Abbreviations

TME: Tumor microenvironment; ESCC: Esophageal squamous cell carcinoma; EC: Esophageal carcinoma; HUVECs: Human umbilical vein endothelial cells; EdU: 5-Ethynyl-2-deoxyuridine; EC9706-Exo: Exosomes secreted from EC9706 cells; TEX: Tumor-derived exosomes; NO: nitric oxide; eNOS: endothelial nitric oxide synthase

Declarations

Acknowledgments

The authors thank the members of our laboratories for their contributions and helpful discussions. The authors also thank Department of Science and Education, Hangzhou First People's Hospital (Zhejiang, China) for their generous help.

Authors' contributions

Tian Wang made substantial contributions to conception and design of. Juan Liao made acquisition of data. Zijie Yang, Weitao Shen, Zhikui Gao and Jiechen Yin made analysis and interpretation of data. Tian Wang have been involved in drafting the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by National Natural Science Foundation of China grants (81872579, 81573108 and 81172747), New Century Excellent Talents in University from Ministry of Education (NCET-13-0124), and Postgraduate Research & Practice Innovation Program of Jiangsu Province (KYCX18_0194).

Availability of data and materials

The dataset(s) supporting the findings of this study are included within the article.

Ethics approval and consent to participate

Not Applicable.

Consent for publication

Not Applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹Key Laboratory of Environmental Medicine Engineering, Ministry of Education, School of Public Health, Southeast University, Nanjing 210009, China;

²Department of science and education, Affiliated Hangzhou First People's Hospital, Zhejiang University School of Medicine

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Figures

Figure 1

Exosomes transport Cy3-miR-21 from EC9706 into HUVECs. a Cy3-labeled miR-21 transferred to recipient HUVECs via donor EC9706 cell-derived exosomes were captured by fluorescent microscope in 24 h. b The relative expression of miR-21 in recipient HUVECs after coculture with EC9706 cells for 24h. *P < 0.05 vs. exo-miR-NC.

Figure 2

miR-21 and EC9706-Exo delivered miR-21 promotes angiogenesis. a Screening for enhanced primary HUVECs tube formation in vitro after treatment with or without miR-21mimic. Scale bar: 200 μm. b, c, d Quantitative evaluation of tube length, number of closed meshes and number of junctions after treating HUVECs with miR-21 mimic or NC mimic. e Screening for enhanced recipient HUVECs tube formation in vitro after coculture with EC9706. Scale bar: 200 μm. f, g, h Quantitative evaluation of tube length,

number of closed meshes and number of junctions in recipient HUVECs after coculture with EC9706. The tube length, number of closed meshes and number of junctions were calculated in three random fields. Results are mean±s.d. *P<0.05 (Student's t-test).

Figure 3

miR-21 and EC9706-Exo delivered miR-21 promotes endothelial cell growth and migration. a, b EdU assays revealed that miR-21 mimics promoted the proliferation of HUVECs (n = 3). c, d Transwell assays demonstrated that miR-21 mimics enhanced the migration ability of HUVECs (n = 3). e, f EdU assays revealed that EC9706-Exo delivered miR-21 promoted the proliferation of HUVECs (n = 3). g, h Transwell assays demonstrated that EC9706-Exo delivered miR-21 enhanced the migration ability of HUVECs (n = 3). *P<0.05 vs. miR-NC/exo-miR-NC.

Figure 4

miR-21 directly targeted PTEN by binding to its complementary sites in 3'-UTR. a Predicted binding sites of miR-21 within the 3'-UTR of PTEN mRNA. b Luciferase reporter gene assay showed that co-transfected with miR-21 mimics significantly repressed the fluorescence ratio of PTEN dependent reporter gene. * $P < 0.05$ vs. miR-NC+WT. c qRT-PCR analysis of PTEN mRNA levels in recipient HUVECs ($n = 3$). * $P < 0.05$. d Western blot analysis of PTEN expression in recipient HUVECs ($n = 3$) and e the corresponding quantitative analysis.

Figure 5

Western blot analysis of p-Akt (Ser473) expression in HUVECs after coculture with donor EC9706 cells.

Figure 6

The detection of NO in the culture supernatant of recipient HUVECs treated with LY294002 and miR-21 inhibitors.